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13. ABSTRACT *(Maximum 200 words)*

Germline mutations in breast cancer susceptibility genes account for 8% of all breast cancer cases. BRCA1 on 17q12 and BRCA2 on 13q12-13 are implicated in 50%-60% of breast cancer families, and they are both likely to also have an impact in sporadic breast cancers and there is evidence for families with linkage to these loci. We propose to ascertain 8 ethically diverse (non-Caucasian) families linked to putative BRCA3 loci on chromosome 8p, and to investigate whether a recently isolated prostate cancer metastases suppressor gene, KAII, is involved in the genetic alterations of inflammatory breast cancer, a particularly aggressive form of breast cancer which accounts for 15% of all cases.

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Introduction

Hereditary breast cancer accounts for 5-8% of breast cancer cases (1). Loci on 8p12-22 are frequently lost in breast tumors and may thus harbor one or more tumor suppressor genes (2). Almost all of breast cancer families studied so far in North America and Western Europe are of Caucasian origin. *We hypothesize that more ethnically diverse families with evidence of autosomal dominant transmission of breast cancer are needed to study loci on 8p12-22.* As current data indicate that *BRCA2* families are rarer than previously predicted, *we hypothesize that a proportion of families found to be unlinked to both BRCA1 and BRCA2 will be linked to recently defined loci on chromosome 8p12-22; as a corollary, the characterization of the 8p-linked familial tumors will help define the limits of the candidate regions on this chromosome.* The specific aims proposed in this application are:

1. To ascertain at least 5 site-specific breast cancer families of diverse ethnic background who are linked to loci on chromosome 8p12-22.

2. To analyze the 8p-linked tumors for LOH at closely spaced markers to help refine the breast cancer candidate region(s).

3. To test families unlinked to *BRCA1*, *BRCA2*, and loci on 8p12-22 for linkage to the *KAI1* gene, a metastases suppressor gene, and to analyze sporadic breast tumors for mutations in *KAI1*.

SIGNIFICANCE AND PRELIMINARY STUDIES

Family collection.

Breast cancer will affect 1,600,000 women world-wide in 1995. In the US alone, 48,000 will die of this disease in 1996 (3). The discovery of breast cancer susceptibility genes has allowed the study of a relatively simple and unique model system for human mammary epithelial carcinogenesis. The tumors suffered by the women belonging to these families are clinically indistinguishable from sporadic tumors. Our laboratory has recently proven that at least 10% of sporadic ovarian cancers undergo inactivation of both BRCA1 alleles.

The scarcity of African American and other minority families in the study of breast cancer susceptibility genes provides the impetus for this application which combines basic research in molecular genetics of breast cancer with a rational attempt at a minority family collection (5-8). The study of the etiology of the widening gap in survival (3) between Caucasian and African American populations requires that future experiments on cancer susceptibility genes be done on an ethnically balanced population of samples. *The lack of availability of these samples and the long lag-time for their acquisition have invariably been the limiting factors. Our proposal intends to contribute shared resources . The Breast Cancer Tissue Core at the University of Michigan (DOD-funded; S. Ethier, Principal Investigator; S. Merajver, co-investigator) provides an independently funded mechanism to freely share these resources for the next 4 years.*

Epidemiological data on family history and breast cancer incidence on Hispanic, Japanese, and Arab women suggests that familial clustering of breast cancer also appears in these populations (9-11). We have been involved for 5 years in an extensive breast cancer family collection and counseling which was at first dedicated to the positional cloning effort of the breast cancer susceptibility gene, *BRCA1* (12,13). *Since 7/1/94, under the direction of Dr. Sofia Merajver, the Breast Cancer Genetics Project has ascertained 114 new breast cancer families. Thirty of those families are in active collection, and 7 have been completely collected.* The principal sources of referrals are: the University of Michigan Breast Care Center (BCC) (450 new breast cancer patients a year), community surgeons, oncologists, and general practitioners; physicians at other research institutions. We have established a successful collaboration with researchers at Henry Ford Hospital (HFH) in Detroit, MI, where a large number (over 150/year) of African American and Hispanic breast cancer patients with a family history are diagnosed and treated. *The family collection at the University of Michigan has proven invaluable in the localization of BRCA1 and in the assessment of familial mutations. The collection provides a renewable source of RNA and*

DNA for functional studies after a tumor suppressor gene is cloned. The families ascertained through the research have the option of gaining knowledge of their risk if they so wish under approved protocols.

Linkage studies to 8p12-22 and other loci.

Fueled by the observation in several laboratories that fewer (10-20%) breast cancer families than previously anticipated (40-50%) are linked to *BRCA2*, other candidate loci such as 8p12-22 are being actively tested, where LOH has consistently been observed above background levels. Forty-seven percent of tumor samples have LOH here and at least one breast cancer family with a LOD (logarithm base 10 of the odds favoring linkage) score of 3.70 for markers in this locus has been described. The study of specific breast cancer families linked to the different loci will provide key information and materials for the understanding of these pathways. *The preliminary studies described here show that there is enough statistical evidence to pursue other tumor suppressor loci as putative familial cancer susceptibility genes on chromosomes 8p12-22 and 11p.*

Loss of heterozygosity (LOH)

Following Knudsen's "two-hit hypothesis" (19), loss of heterozygosity (LOH) is generally a marker of the presence of a tumor suppressor gene. S. Merajver has studied LOH in familial and sporadic breast and ovarian tumors for the last 4 years (4,12). *These experiments demonstrate that LOH at closely spaced, ordered markers on a tumor suppressor candidate region can define a minimum region of overlap and help localize the desired transcript. This experimental design can be extended to other tumor suppressor regions, such as 8p12-22.*

Mutation analyses of familial and sporadic specimens.

Advances in familial cancer syndromes are also relevant to sporadic carcinogenesis. For example, the PI and others have recently shown that *BRCA1* is implicated in sporadic carcinogenesis (4). Dr. Merajver's laboratory was the first to report 4 somatic mutations (12). The initial screen for mutations was performed by a modification of single-strand conformation polymorphism (SSCP) technique. These *experiments show that even for a fairly large gene such as BRCA1, thorough, semi-automated, batch-mode mutation analyses can be performed efficiently and rapidly with a combination of SSCP and direct sequencing of familial and sporadic tumors. Even in the complicated situation posed by BRCA1, these methods have proven effective; we believe that it is not overly optimistic to predict that they will serve us well for tumor suppressor genes on 8p12-22.*

SUMMARY OF PRELIMINARY STUDIES .

The PI has performed family collection studies (13), directs a high-risk breast cancer clinic where family members are counseled, has conducted detailed mapping studies of sporadic tumors by LOH (12), and has ascertained that the mutations of the first described familial breast cancer gene (*BRCA1*) are present in sporadic tumors (4). Research in all these areas has been published in the peer-reviewed literature. (4,12,13)

Body

The work performed in Dr. Merajver's laboratory under this grant is part of a multifaceted collaborative effort to understand the molecular genetics of familial and sporadic breast cancers. The laboratory efforts in this regard are now proceeding along 3 well-defined lines of investigation, as follows:

(1) Family linkage studies to 8p, BRCA1, and other genes.

The work on 8p involving families and tumors has progressed as follows, in accordance to the specific aims of the project.

We have proceeded with our studies of families with potential linkage to *BRCA3* putatively assigned to chromosomal region 8p12-22. In this project we have also ascertained 8 families in which affected individuals appear to share disease-associated 8p haplotypes. We are building a collaboration with investigators at Johns Hopkins

University Oncology Center in the search for breast cancer genes on 8p. We are planning on sending these investigators samples from these select families for further studies with unpublished markers in the region, and we will share with them the studies of mutations of any new genes identified. We have also ascertained 23 tumors with deletions at 8p of various sizes which we will share collaboratively with the Hopkins group.

We have expanded our family resources by continuing to build a cancer genetics network with several key institutions in the State. In particular, we have now joined forces and protocols with the Karmanos cancer Institute and Butterworth Hospital in Western Michigan. We continue to utilize the community resources we have gathered, and right now we have as many families as we can study (even with our collaborators) in the next year, approximately as planned in the SOW.

The collection of blood specimens has been undertaken in the manner in which we have proceeded so far, under an IRB-approved protocol. In brief, after signing informed consent, we draw 3 lavender-top tubes (5 cc each) for DNA and 2 green-top tubes (for lymphocyte immortalization). The DNA extraction and immortalization protocols are described below. Pre-paid mailing kits or travel to the family's domicile are used for remote specimens. This latter method is very cost-effective, allows for the establishment of close rapport with the family, and provides for an opportunity to answer questions regarding the research and breast cancer. The familiar specimen collection proceeded at an extremely fast pace, and, at present, except for striking families, we have more samples that we can realistically fully analyze in the next 8-12 months, so we are concentrating on the analyses primarily. We have extracted the DNA performed the LOH experiments on 8p. We are also poised to proceed in an expedient manner were the actual BRCA3 gene to be isolated by other labs in the near future. This portion of aim 1 is ahead of schedule as per the SOW.

(2)Linkage analyses.

Linkage analysis is performed using the method of LOD scores (14-16). Calculations are carried out with the programs MENDEL (15) and LINKAGE (16). All families will be screened for linkage to *BRCA1* with *D17S855* and *D17S1323*, two intragenic polymorphic markers, and *BRCA2*., the latter with *D13S221, D13S260, D13S267, and D13S263.* Following Kerangueven et al (2), we will use the following panel of markers on 8p12-22 (Table 1).

For each family not linked to either *BRCA1*, or *BRCA2*, or 8p12-22, rapid SSCP analyses of 2 affected individuals with age of onset of cancer under 50 were conducted on the *KAI1* gene on 11p11.2 (17). The primers for this gene were designed and synthesized, and tested on DNA from lymphocytes and archival materials. The results, however, were negative, and no alterations were found. For this reason, we modified our course and proceeded to actually isolate more genes involved in breast cancer progression and aggressive phenotypes, as summarized below. This entirely new area of investigation has proven extremely promising and successful, and was entirely spurred by this negative result..

TABLE 1. 8p markers to be used in this study ordered from centromere to telomere

Tumor LOH	Linkage studies
D8S137	D8S137
NEFL	*NEFL*
D8S259	
D8S133	D8S133
D8S258	
LPL	
D8S261	D8S261
D8S265	

The 8p markers have all been synthesized, tested on trial paraffin specimens (we are always extremely frugal with our valuable familial specimen resources), and optimized for amplification of paraffin-embedded material. The analyses of 50 familial and over 40 sporadic specimens along the 8p12-22 region will continue for the next 0.6-0.8 years, as per the SOW.

(3) Additional Projects. (Undertaken under this grant due to auspicious circumstances which modified and expanded the course of the research).

(3.1) BRCA2.

We ascertained 4 new BRCA2 families. We recognized 1 novel BRCA2 mutation on exon 11 which occurs in a breast/ovarian family. This is especially important in light of the phenotype-genotype correlation reported between exon 11 and the risk of ovarian cancer, as this mutation further reinforces that relationship.

(3.2) DIFFERENTIAL EXPRESSION OF GENES IN THE SUM149 CELL LINE WHICH MAY CONTRIBUTE TO THE PATHOGENESIS OF INFLAMMATORY BREAST CANCER.

Inflammatory breast cancer affects approximately 6% of all breast cancer patients in the United States. The disease is characterized by a dark red and swollen appearance of the skin overlying the breast which is caused by the infiltration and subsequent blockage of the lymph vessels by the carcinomatous emboli. Clinically, it is a rapidly growing tumor which is classified at diagnosis as a T4d (stage IIIb) carcinoma. Clinical evidence suggests that nearly all of women with inflammatory breast cancer have axillary nodal involvement at the time of diagnosis. Furthermore, it is estimated that at the time of diagnosis, 36% of women with inflammatory breast cancer have distant metastases; this number increases significantly within one year. Because of the prevalence of nodal and metastatic involvement, prognosis for long-term disease-free survival is guarded. At present, the genetic factors and pathways which underlie the pathogenesis of inflammatory breast cancer have yet to be elucidated.

To address this question, our laboratory has isolated and identified genes which are differentially expressed in normal versus inflammatory mammary tumor cells. Messenger RNA isolated from the SUM149 cell line, which was developed at the University of Michigan from an aggressive primary infiltrating ductal carcinoma, was compared with mRNA from the same patient's own lymphocytes and from two normal mammary epithelial cell lines. Comparison of the cDNAs from this system was accomplished using the differential display technique.

All differentially expressed messages were cloned and sequenced. Nine genes were shown to be expressed in the normal cells but not in the tumor cells. Of these genes, three out of nine are known genes; an olfactory receptor gene, *H-NUC* and *HMG-CoA reductase*. The protein products of the latter two genes have previously been implicated in breast cancer progression and the control of cell growth. H-NUC is a protein which is associated with the hypophosphorylated form of the tumor suppressor retinoblastoma protein. An increase in cell growth following loss of HMG-CoA reductase activity along with concurrent addition and stimulation of cells with growth promoters such as estrogen or epidermal growth factor has been previously demonstrated in some breast cancer cell lines. Additional breast tumor samples are being tested for mutations in these genes by SSCP analysis. The olfactory receptor genes are located on chromosome 17p13.1-17p13.3, an area which contains known tumor suppressor genes. No loss of heterozygosity was seen in the SUM149 cells at this or three other regions of chromosome 17, including *TP53* and *BRCA1*. The remaining six genes expressed exclusively by the normal mammary cells are novel and do not match any published sequence in the EST database. An additional group of four novel genes has been shown to be exclusively expressed in the tumor cells and match published sequences in the EST database.

(3.3) IDENTIFICATION OF GENES DIFFERENTIALLY METHYLATED IN INFLAMMATORY BREAST CANCER.

Inflammatory breast cancer affects only about 6% of breast cancer patients, but its metastatic potential and clinical manifestations contribute to its poor prognosis. To date, very few molecular mechanisms have been suggested which contribute to the progression of this disease. To identify one potential differential mechanism, we studied the methylation alterations of genes of the Sum 149 cell line (derived from a primary inflammatory breast cancer) as compared to normal human mammary epithelial cells, since altered gene methylations has been observed in cancers of many types. Fragments that showed differential methylation were cloned, sequenced, and used as probes in the Southern blot hybridization to confirm differential methylation. Ten of the thirteen candidate genes were found to be hypomethylated while the remaining three were hypermethylated in the tumor DNA. Three of the ten hypomethylated regions showed significant localization to chromosome 7. A hypomethlyated gene encoding exon 2 of the human frataxin gene, which is known to be flanked by a cluster of CpG islands was also isolated. Mutations in this gene lead to Friedreich's ataxia an autosomal recessive neurodegenerative disorder. The remaining hypo and hypermethylated genes showed significant matches to a variety of sequences in the EST database. These results suggest that differential methylation of genes is a potentially important phenomenon in the pathogenesis of inflammatory breast cancer. Further investigations are underway to assess the functional significance of these changes.

List of Publications and Abstracts directly supported or partially supported by this grant (1996-1997).

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16. LeClaire V, Taylor K, Merajver SD: A Mandate for the Future: The Multidisciplinary Cancer Risk Counseling Program. Michigan Nurse's Association Annual Conference, October 7, 1997.

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Conclusions

The work on 8p continues to prove encouraging that an important BRCA3-like gene is harbored by that chromosomal region. Our laboratory investigations of differentially expressed genes in aggressive breast cancers have led, in the last year, to striking progress. We are now poised to further study the newly isolated genes. We hope to devote the remainder of the grant period to this task, and to seek other sources of funding to continue this work in the near future.

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